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THE CELLULOLYTIC SYSTEM OF *TALAROMYCES EMERSONII*

IDENTIFICATION OF THE VARIOUS COMPONENTS PRODUCED DURING GROWTH ON CELLULOSIC MEDIA

ANTHONY McHALE and MICHAEL P. COUGHLAN *

Department of Biochemistry, University College, Galway (Republic of Ireland)

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***Talaromyces emersonii*, a thermophyllic fungus was grown on cellulose/corn steep liquor/NH_4NO_3 medium. The kinetics of growth and extracellular cellulase production were measured. The enzyme system was found to be comprised of four to five forms of exo-β-1,4-glucanase (cellobiohydrolase; 1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91), at least two forms of endo-1,4-β-glucanase (1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4) and three enzymes exhibiting β-glucosidase (cellobiase; β-D-glucoside glucohydrolase, EC 3.2.1.21) activity. One of the latter, termed β-glucosidase III, is induced concurrently with the cellulases but disappears from the medium because of the low pH that develops during growth. The cellulases by contrast are more acid-stable. Later in the growth cycle a second form of β-glucosidase, termed β-glucosidase I, accumulates. An intracellular β-glucosidase, β-glucosidase IV, was also detected. The possible functions of these enzymes are discussed.**

Introduction

If enzymic hydrolysis of cellulose is to be a commercial proposition it is essential that highly active enzyme be available at low cost. *Trichoderma viride* has long been regarded as the best source of such enzymes [1,2]. Indeed cellulase-hyperproducing mutants of this organism have now been isolated [3,4]. The thermophyllic fungus, *Talaromyces emersonii*, when grown on cellulosic media produces an extracellular enzyme system capable of catalyzing extensive degradation of crystalline substrate [5–8]. Moreover, such activity is achieved in about 50 h, whereas production of maximal activity by *T. viride* takes 5–8 days [9,10]. Thus, while *T. viride* provides the more powerful enzyme, the possibility of lower fermentation costs and the fact that it is a thermophyle suggest that *T. emersonii* should also be subjected to strain selection and mutation techniques. However, before we can isolate more efficient mutants it is important that we understand the nature of the cellulolytic system of the wild type in more detail.

Materials

Chemicals were obtained from the following suppliers: Solka Floc (BW. 40; purified ball-milled spruce cellulose) was from Brown & Co., Berlin, NH, U.S.A.; corn steep liquor from Biocon Ltd., Cork, Republic of Ireland; sodium dodecyl sulphate (SDS) *p*-nitrophenyl-β-D-glucoside and low viscosity carboxymethyl cellulose from Sigma (London) Chemical Co.; dinitrosalicylate and *N,N,N',N'*-tetramethylenediamine (TEMED) from BDH.

* To whom correspondence should be addressed.

Methods

Organism. Talaromyces emersonii CBS 814.70 obtained from Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands was grown at 45°C as described above [5,7] on medium containing 2% (w/v) Solka Floc/0.5% (w/v) corn steep liquor/1% (w/v) NH_4NO_3.

Enzyme assay. Total cellulase activity was determined by measuring the release of reducing equivalents (with glucose as standard) by the dinitrosalicylate method [11] following incubation of 0.2 ml aliquots of culture filtrate with 30 mg filter paper in 0.1 M sodium acetate buffer, pH 5.0 (final volume 2 ml) for 2 h at 60°C. Endocellulase (CM-cellulase or C_x) activity was measured as above with 6% (w/v) carboxymethyl cellulose as substrate. At 33°C this activity was constant up to 0.66 mg glucose reducing equivalents [8]. β-Glucosidase activity was measured as described by Wood [12] but using *p*-nitrophenyl-β-D-glucoside as substrate.

Electrophoresis. Samples of culture filtrate or of homogenized mycelium were harvested at the appropriate time and were subjected to gradient polyacrylamide gel electrophoresis [13] but omitting the SDS. β-Glucosidase activity was located by immersing the gels in 0.1 M sodium acetate buffer, pH 5.0/20 mM *p*-nitrophenyl-β-D-glucoside or cellobiose followed by a light wash with water and then immersion in 0.4 M glycine-NaOH buffer, pH 10.8 or glucose oxidase reagent, respectively. Endocellulases and exocellulases (C_1 or cellobiohydrolase) were separated by gel electrophoresis in 5% polyacrylamide gels using the buffer system described by Reisfeld et al. [14]. The cathode was at the top and the anode at the bottom. Gels were pre-run for 2 h at 5 mA/tube. The sample was then applied in 10% (w/v) sucrose in the electrode buffer. Bromocresol purple (0.04%) was used as marker dye. Gels were stained for protein with 0.1% (w/v) Coomassie blue in 50% (w/v) trichloroacetic acid, and for glycoprotein with the periodic acid-Schiff reagent of Zacharius et al. [15]. The exact location of endocellulase and exocellulase activities was determined as follows. The gels were exposed briefly to 50% trichloroacetic acid so as to denature protein at the surface and render visible the precise location of each protein band. The gels were washed with distilled water, the bands were excised and the protein eluted by soaking overnight in 0.1 M sodium acetate buffer, pH 5.0. Despite denaturation of the surface protein by trichloroacetic acid sufficient enzyme remained active in the centre of each zone to allow identification as described earlier [8]. Some samples of culture filtrate were subjected to two-dimensional electrophoresis-polyacrylamide gel electrophoresis as above in the first dimension followed by equilibration in 62.5 mM Tris-HCl buffer, pH 8.3/2.3% (w/v) SDS/5% (w/v) mercaptoethanol/10% (w/v) glycerol and then SDS-polyacrylamide gradient gel electrophoresis [13]. Coomassie blue-stained gels were scanned at 550 nm using a Pye Unicam SP 800 especially adapted for the purpose.

Results and Discussion

Fermentation parameters. The results of a typical 10 l fermentation on cellulose/corn steep liquor/NH_4NO_3 medium are shown in Fig. 1. Cell protein increases rapidly for about 45 h and then decreases presumably because of lysis of the cells. Meanwhile, in confirmation of our earlier findings [7], the pH of the medium rises slowly from 5.0 to about 5.7 at 25 h and then drops sharply to about 3.2 at 45 h before again increasing slowly to around pH 4 at 80 h. This pH profile is very similar to that produced by *T. viride* during growth on $(NH_4)_2SO_4$-containing media [9,16]. In this case the sharp drop in pH was said to be due to increasing assimilation of nitrogen which in turn was consequent on the increasing uptake of glucose as cellulose was hydrolyzed. The uptake of nitrogen as NH_3 left an excess of H^+ in the medium [9]. The production of organic acids during growth might provide an alternative explanation for the observed pH profile. Whatever the reason the fact remains that pH has profound effects on the activity and stability of the extracellular enzymes (see below and Ref. 17). Total cellulase activity in the culture filtrate lags a little behind cell growth but essentially reaches a maximum at about the same time, i.e., 48–50 h. The lag in the appearance of cellulase in the culture filtrate may be explaned in part by the fact that enzyme secreted by the organism in the early stages of growth adsorbs to the cellulose which is still present at high concentration (see e.g., Ref. 18). Thus, the real lag in enzyme production may be considerably less if one measured all extracellular cellu-

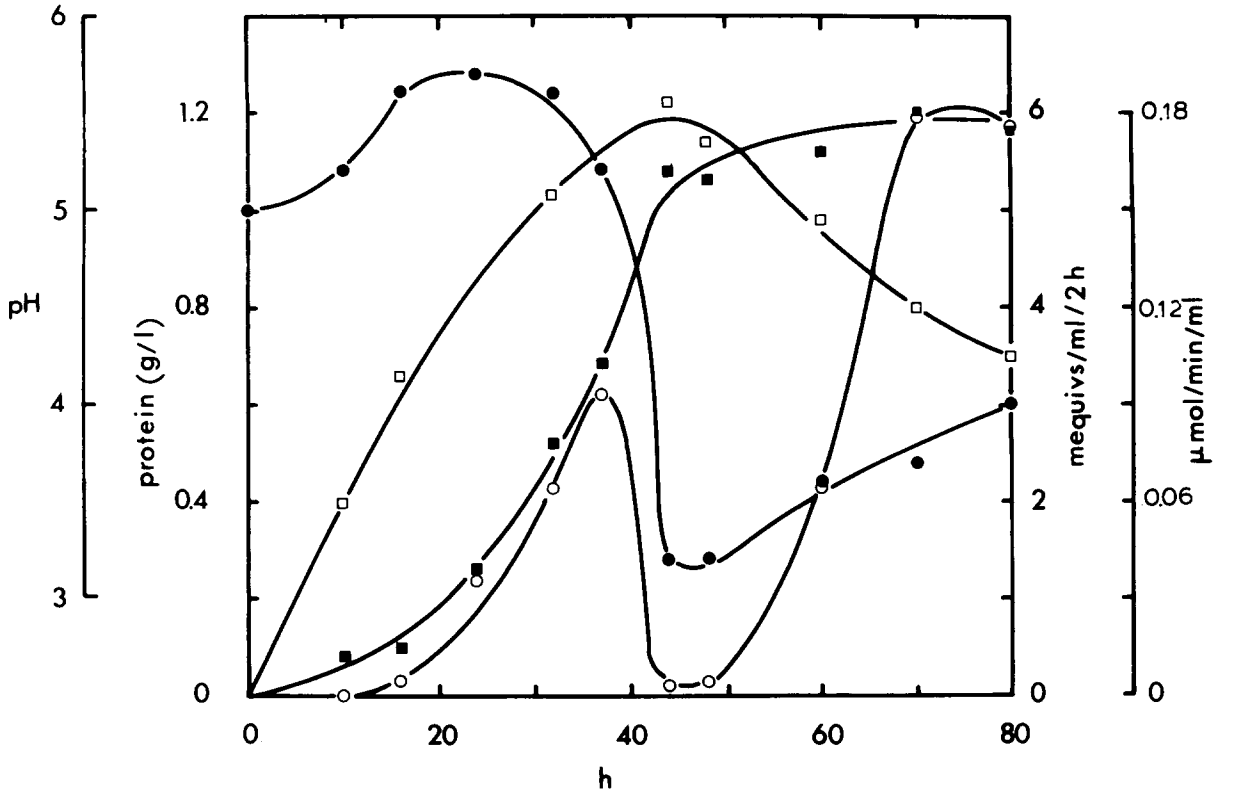

Fig. 1. Growth and enzyme production by *T. emersonii* in liquid medium. A 10 l cellulose/corn steep liquor/NH_4NO_3 fermentation was run at 45°C as described earlier [5]. Samples of mycelia obtained at intervals were solubilized in NaOH [5] and protein content (□) measured by the method of Hartree [24]. The pH (•), total cellulase activity (▪) and β-glucosidase activity (○) of the culture filtrate were also measured at various times during the fermentation.

lase activity, i.e., soluble plus adsorbed. Note however that there was no appreciable lag in enzyme production by *T. emersonii* when grown on cellulose/peptone medium [5]. Sternberg [9] has demonstrated that with spore inocula the inclusion of peptone in the medium reduces the lag in enzyme production by *T. viride*. While we have not used spore inocula it is likely that the effects of peptone are due to the fact that it is more readily assimilated by the organism than ammonia. However, one should bear in mind the possibility that peptone may also facilitate release of the enzyme from the organism. Fig. 1 also shows two peaks of β-glucosidase activity during the growth cycle, the first at about 36 h and the second between 70–80 h. Possible reasons for these findings are discussed later. Meantime we were interested in seeing whether more than one type of β-glucosidase was produced by this organism and, if so, what was the pattern of appearance of each individual form. We were also interested in identifying the other components of the cellulase system. To this end samples of culture filtrate and of mycelia, harvested at various intervals during a typical fermentation, were subjected to polyacrylamide gel electrophoresis in normal and gradient gels.

Polyacrylamide gel electrophoresis of such samples gave the patterns illustrated in Fig. 2a. Each band was excised, eluted and assayed as described in methods. The results indicated that the four or possibly five bands nearest the anode exhibited exocellulase activity. The next two bands showed endocellulase activity while none of the remaining bands of protein had the ability to hydrolyze either carboxymethyl cellulose or phosphoric acid-swollen cellulose. The intensity of staining of the various bands suggest that endo- and exocellulase activities increase in parallel

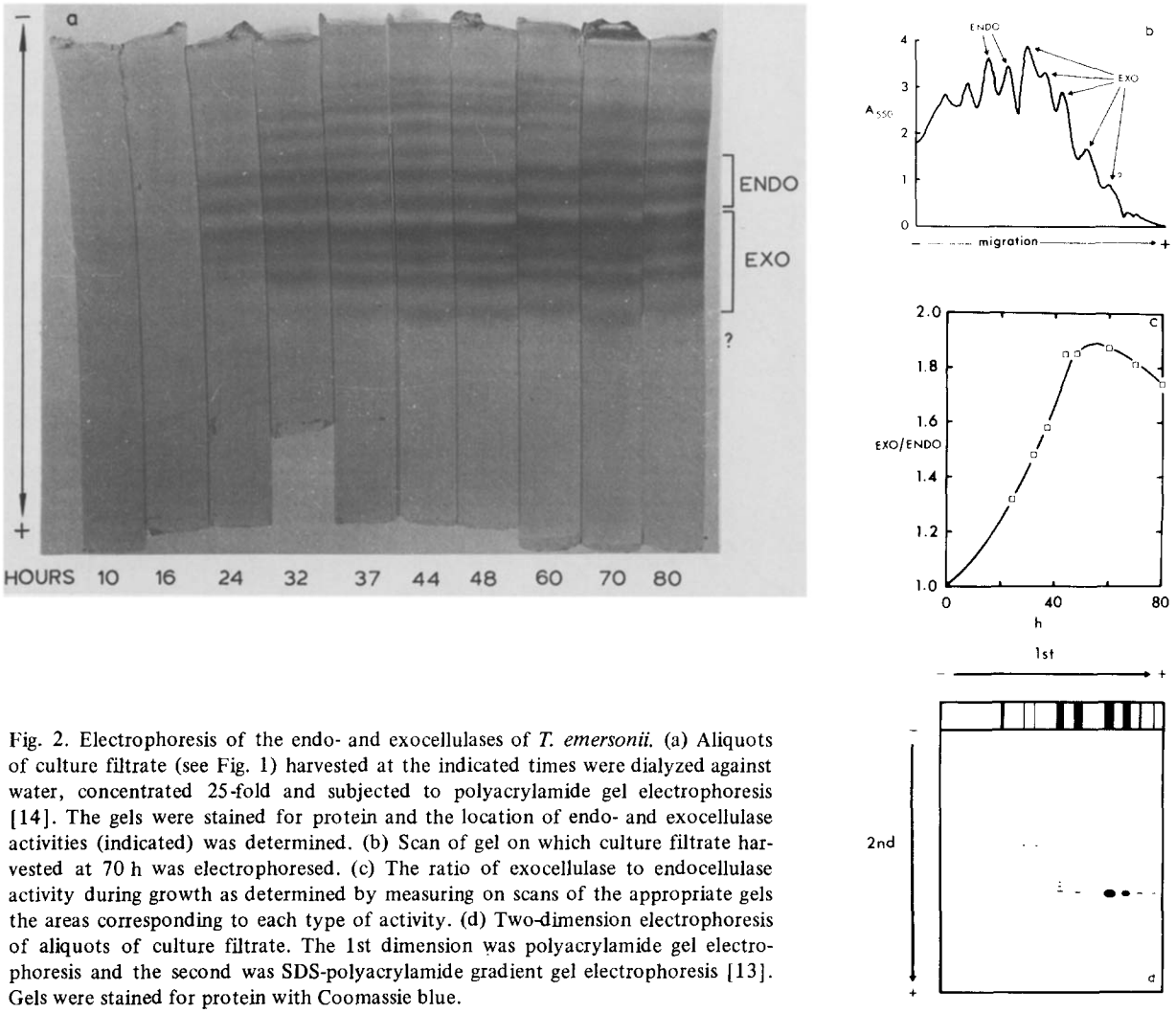

Fig. 2. Electrophoresis of the endo- and exocellulases of *T. emersonii*. (a) Aliquots of culture filtrate (see Fig. 1) harvested at the indicated times were dialyzed against water, concentrated 25-fold and subjected to polyacrylamide gel electrophoresis [14]. The gels were stained for protein and the location of endo- and exocellulase activities (indicated) was determined. (b) Scan of gel on which culture filtrate harvested at 70 h was electrophoresed. (c) The ratio of exocellulase to endocellulase activity during growth as determined by measuring on scans of the appropriate gels the areas corresponding to each type of activity. (d) Two-dimension electrophoresis of aliquots of culture filtrate. The 1st dimension was polyacrylamide gel electrophoresis and the second was SDS-polyacrylamide gradient gel electrophoresis [13]. Gels were stained for protein with Coomassie blue.

during the growth cycle and reach a maximum between 48 and 60 h. This pattern is consistent with the kinetics of appearance of cellulase activity in the culture filtrate (Fig. 1). However, scans of the gels (an example is shown in Fig. 2b) and measurement of the areas corresponding to both types of activity indicate that the exocellulase/endocellulase activity ratio in culture filtrate increases up to about 50 h before decreasing slightly (Fig. 2c). This method of comparing activities is relatively crude. Thus, more detailed studies would be required to determine whether in this organism induction of the synthesis of endo- and exocellulases is coordinated or not, whether both types of enzyme are secreted into the medium at comparable rates and whether the adsorption of each type of enzyme to undigested cellulose is the same or not. Fig. 2d shows the results of two-dimensional electrophoresis of a sample of culture filtrate harvested at 48 h. The four or five exocellulases which separate on the basis of charge/size ratio on normal polyacrylamide gel electrophoresis (1st dimension) migrate at the same rate on SDS-polyacrylamide gradient gel electrophoresis (2nd dimension). Clearly, the individual exocellulases have similar if not identical molecular weights but differ in effective charge. Fig. 2d also suggests that as many as five different

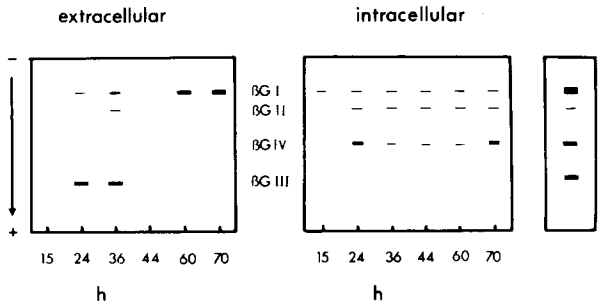

Fig. 3. Gradient gel electrophoresis of the extracellular and intracellular β-glucosidases of *T. emersonii*. (a) Samples of culture filtrate obtained at various times during fermentation were dialyzed, concentrated approx. 25-fold, subjected to gradient gel electrophoresis and stained for β-glucosidase activity. (b) Samples of mycelia harvested at different times were washed and homogenized and aliquots of the concentrated supernatant were subjected to electrophoresis and stained for activity. (c) Shows that a mixture of the four β-glucosidases migrate as before on gradient gel electrophoresis.

forms of endocellulases may be present in culture filtrate. The above results may indicate that true isoenzymic forms of these cellulases exist. Alternatively, the various forms may be due to differential glycosylation (they all stain for carbohydrate) or aggregation of the same primary structures.

Samples of culture filtrate harvested at various times during a typical fermentation were subjected to gradient gel electrophoresis and stained for β-glucosidase activity. Three forms of β-glucosidase were found in the filtrate (Fig. 3a). These we call in order of decreasing molecular weight β-glucosidase I, II and III. The latter is the predominant form early in the fermentation during which time only traces of β-glucosidase I and II are present. At 44 h no band staining for β-glucosidase activity was evident. This is consistent with the virtual lack of such activity on assaying samples of filtrate harvested at this time (see Fig. 1). β-Glucosidase I is the sole form of the enzyme present in culture filtrate in the latter stages of the fermentation at which time β-glucosidase activity is greatest (see Fig. 1). In addition to these forms of β-glucosidase is another, intermediate in size between β-glucosidase II and III, which we have named β-glucosidase IV. This form which is found only within the cell is present throughout the growth cycle but especially at the beginning and end (Fig. 3b).

Fig. 3, therefore, clearly shows that a different form of the enzyme predominates or is exclusive to each peak of β-glucosidase activity in the culture filtrate during growth (see Fig. 1). But why do two peaks of such activity exist? The answer to this question may include one or more of the following possibilities. Thus, the enzymes may be fundamentally different. The conditions that induce the synthesis of each form may differ. Finally, the alteration in the pH of the medium during growth may have a differential effect on activity or stability.

The first possibility would appear to hold good. Examination of substrate specificity [17] shows that while β-glucosidase I is a true cellobiase β-glucosidase III may more properly be considered as a β-1,4-glucose hydrolase (glucohydrolase). Thus, it removes glucose residues from β-1,4-linked polymers of glucose at least up to cellohexaose. Moreover, at the same substrate concentrations the rate of hydrolysis increases 30-fold in going from cellobiase to cellohexaose and the enzyme has 26-fold greater affinity for cellotetraose than for cellobiose [17]. Thus, while β-glucosidase I and β-glucosidase III exhibit β-glucosidase (cellobiase) activity they would appear to have fundamentally different roles in vivo.

With respect to the conditions necessary for induction of the synthesis of β-glucosidase I and β-glucosidase III two observations by Sternberg and Mandels [19,20] are pertinent. These workers showed that sophorose, a β-1,2-linked disaccharide of glucose, not only induced the synthesis of cellulases by *T. reesei* but also repressed the synthesis of β-glucosidase. Fig. 1 shows that the appearance of β-glucosidase III and of cellulase in the medium parallel one another, whereas the increase in the amount of β-glucosidase I present only begins when cellulase has already reached its maximum value (c. 48 h) and peaks some 20–30 h later than cellulase. The true inducer of cellulase synthesis may, like sophorose, also repress the production of β-glucosidase. These observations lend further support to our thesis that β-glucosidase III is in reality an exo-β-1,4-glucohydrolase i.e., that it be classified as a cellulase. Moreover, it should be noted that β-glucosidase I but not β-glucosidase III is produced by *T. emersonii* when grown on glucose or cellobiose rather than cellulose (see below).

The discussion above may explain the differential onset of synthesis of β-glucosidase I and β-glucosidase

III but does not explain the disappearance from the medium of β-glucosidase III once synthesized and released. The answer to this latter question was provided by examination of the stability of β-glucosidase III as a function of pH and at 45°C, the temperature at which fermentation is carried out. At pH values around five the enzyme was relatively stable but at pH 3 and 45°C, the conditions obtained at 45 h in growth cycle (Fig. 1), the half-life was less than 2 min [17]. Since activity corresponding to β-glucosidase III did not return when the pH and climbed back to 4 (see Figs. 1 and 2) it is obvious that the conditions necessary for its synthesis no longer obtained and that enzyme released at earlier stages of growth had been denatured. Analogous observations on the production of a β-glucosidase by *T. reesei* have been made by Sternberg [9,16]. This enzyme is inactivated under the acid conditions that develop in the medium during growth of the organism on cellulose. With appropriate control of pH inactivation of β-glucosidase is obviated and activity accumulates in the medium [9,16].

As a further check on the theory that the loss of β-glucosidase III from the growth medium was due to the attainment of low pH and to see if the observations above on *T. reesei* also obtained in the case of *T. emersonii* we examined the kinetics of enzyme production in pH-controlled and uncontrolled flask cultures. The results (data not shown) may be summarized as follows. Thus, in cellulose/corn steep liquor/NH_4NO_3 medium, in which the pH was controlled at 5 by the inclusion of 0.1 M sodium citrate buffer, β-glucosidase III was present throughout the growth cycle. β-Glucosidase I, present in small amounts in the early stages of growth began to increase after 50 h. However, the total β-glucosidase activity in the pH 5-controlled medium at any time during the 100 h observation period was not significantly greater than that in the uncontrolled medium. When the pH of the medium was controlled at 3.6 β-glucosidase III was not detected in the culture fluid at any stage during growth, whereas β-glucosidase I behaved qualitatively as before. When cellulose/peptone medium was used the pH increased from 5 to about 6.9 during the growth cycle (see Ref. 5). In this case β-glucosidase III was also present in the culture filtrate throughout but again β-glucosidase I was present in significant amounts only at later stages of growth. Total cellulase activity in medium controlled at pH 5 was the same as that obtained without pH control (see also Ref. 5) and was twice that obtained in peptone medium or in pH 3.6-controlled medium. Endocellulase activity in culture filtrate was as follows: uncontrolled > pH 5-controlled > pH 3.6-controlled > peptone.

When cellulose was replaced by cellobiose or with glucose in the usual growth medium only trace amounts of total cellulase activity was found while endocellulase and β-glucosidase III activities were not detectable at any stage. By contrast, the levels of β-glucosidase I in culture filtrate increased as growth proceeded but never amounted to more than one-fifth of that obtained with cellulose-containing medium. These then would appear to be the constitutive levels of β-glucosidase I. Moreover, the above results show that cellulose or a derivative of cellulose other than cellobiose induces the synthesis of the cellulases and of β-glucosidase III. In keeping with the observations of Sternberg and Mandels [20] on sophorose we could perhaps suggest that the true inducer not only stimulates the synthesis of the above enzymes but also represses the production of β-glucosidase I. Accordingly, synthesis of β-glucosidase I would get under way only when the above repressor had been consumed, as sophorose is ultimately consumed by β-glucosidase [20], or converted to an inducer or when the appropriate inducer had been produced by some other route.

Conclusion

The appearance of cellulase enzymes in the culture fluid of *T. emersonii* is not due to lysis of the cells. On the contrary, it is a true extracellular enzyme system. Note that maximum cellulase activity is achieved at about 50 h, i.e., prior to the onset of the decrease in cell protein (Fig. 1). However, cellulase synthesis and secretion does slow down or stop at this stage presumably because the organism is running out of nutrient. Microscopic examination show the presence of some spores at this stage. We have shown in a previous paper [8] that the ability of this organism to hydrolyze crystalline cellulose results from the combined actions of endocellulases, exocellulases and β-glucosidases. Thus, the slight decrease in extracellular cellulase activity at about 60 h (Fig. 1) is due to

the lacuna between the disappearance of β-glucosidase III and the appearance of significant quantities of β-glucosidase I. If the *T. emersonii* system is to be used for practical saccharification of cellulosic materials the levels of β-glucosidase activity would have to be improved upon considerably (see Refs. 21, 22). Gong and Tsao [23] have put forward a model for the regulation of cellulase biosynthesis in which an intracellular β-glucosidase has a central role. Perhaps such a role is among the functions of the intracellular β-glucosidase, β-glucosidase IV, of *T. emersonii*.

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